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(21) International Application Number: PCT/DK94/00122 (22) International Filing Date: 24 March 1994 (24.03.94) (30) Priority Data: 382/93 31 March 1993 (31.03.93) DK (71) Applicant (for all designated States except US): NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsvaerd (DK). (72) Inventors; and (75) Inventors/Applicants (for US only): JØRGENSEN, Tony [DK/DK]; Tranemosevej 76, DK-2750 Ballerup (DK). PEDERSEN, Anders, Hjelholt [DK/DK]; Nybro Vænge 58, DK-2800 Lyngby (DK). (74) Common Representative: NOVO NORDISK A/S; Corporate Patents, Novo Allé, DK-2880 Bagsvaerd (DK).		(81) Designated States: AU, CA, CN, CZ, FI, HU, JP, KR, NO, PL, RU, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i>
(54) Title: PURIFICATION OF FACTOR VII (57) Abstract Activation and degradation of FVII during purification by means of a number of chromatographic purification steps is controlled by the presence of Zn ⁺⁺ at least in one of the purification steps.		

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PURIFICATION OF FACTOR VII

Technical Field

The present invention is related to a method for controlled activation and degradation of Factor VII.

5 Background Art

Coagulation Factor VII (FVII) is a vitamin K dependent serine protease playing a key role in the extrinsic pathway of blood coagulation. It is synthesized in the liver and secreted into the blood where it circulates as a single-chain glycoprotein (zymogen) with a molecular weight of about 50,000. In its activated form, FVIIa, the protease
10 catalyzes the activation of two other vitamin K dependent coagulation factors of the serine protease family, Factor IX (FIX) and Factor X (FX).

Activated FX (FXa) will then convert prothrombin into thrombin. Thrombin will then convert fibrinogen into fibrin, the major constituent of the clot.

Factor VII can be purified from plasma and activated into Factor VIIa by the methods
15 described by Broze and Majerus, J.Biol.Chem. 255 (4) (1980) 1242 - 1247 and Hedner and Kiesel, J.Clin.Invest. 71 (1983) 1836 - 1841.

Factor VIIa may also be produced by recombinant DNA-technology by culturing in an appropriate medium mammalian cells transfected with a DNA-sequence encoding Factor VII, isolating the protein produced and activating said protein to Factor VIIa
20 (vide European patent application No. 86302855.1).

Factor VIIa may be used in treating patients who have developed inhibitors to Factor VIII (Hedner, U. and Kiesel, W, J.Clin.Invest, 71 (1983) 1836 - 1841) and for the

treatment of patients suffering from bleeding disorders such as platelet disorders including thrombocytopenia, von Willebrand's disease and others typically present in association with severe tissue damages (European patent application No. 86309197.1).

5 During purification of the plasma-derived bovine protein (Radcliffe & Nermerson, J.Biol.Chem. 250 (1975) 338 - 395) and the human recombinant protein (Thim et al., Biochemistry 27 (1988) 7785 - 7793), FVII was activated into the two-chain form by hydrolysis of the Arg₁₅₂-Ile₁₅₃ bond. The activation of FVII is greatly enhanced by adsorption on anion exchangers (diethylaminoethyl or trimethylaminoethyl modified
10 polymeric gel matrices) (A.H. Pedersen et al., Biochemistry 28 (1989), 9331-9336). The mechanism by which the FVII activation is enhanced is not known. In addition to the activation, a fraction of the FVIIa molecules is cleaved primarily at positions 290 and/or 315 by autodegradation (E.M. Nicolaisen et al., FEBS Lett. 317 (1993) 245-249). Such degradation products are inactive molecules and their occurrence
15 in the Factor VIIa preparation will lead to a lower specific activity of the final preparation. Furthermore, the amount and nature of the degradation products may vary from one production batch to another giving rise to preparations with a variable content of biologically active Factor VIIa. A content of degradation products in the final preparation may trigger the immune system of the patient. Readministration may
20 then result in allergic reactions, which in severe cases may have a lethal course. Patients may also develop high titers of antibodies against Factor VIIa rendering subsequent treatment difficult or ineffective.

In order to prepare a purified FVIIa product with a low content of degradation products it is essential to control the activation and impede degradation during the
25 purification process and subsequent processing. In contrast to FVIIa the single chain form, FVII, is resistant to or much less prone to cleavage in the heavy chain. It might therefore be an advantage to purify FVII in its single chain form.

It is therefore the purpose of the present invention to provide a purification process for FVII by which activation and degradation is avoided or kept at an acceptable low degree with the purpose of providing a homogeneous product of high purity which can then be activated into FVIIa in a further step in high yields to give a uniform and 5 homogeneous product with high specific activity.

It is known that zinc ions inhibit the amidolytic and proteolytic activity of recombinant FVIIa (Pedersen, A.H. et al., Thrombosis and Haemostasis, 65 (1991), 528 - 534). It has now surprisingly been found that addition of zinc ions can be used to control the autoactivation of FVII and to impede the degradation of FVII/FVIIa during 10 purification by means of chromatographic column materials.

Description of the Invention

In its broadest aspect the present invention is related to a method for controlled activation and degradation of FVII during purification whereby a solution of Factor VII is subjected to a number of chromatographic purification steps wherein Zn^{++} is 15 present at least in one of the purification steps.

In a more narrow aspect the present invention is related to a method for controlled activation and degradation of FVII wherein a solution of FVII is applied to a number of anion exchange and immuno affinity columns.

In a preferred embodiment of the present invention the FVII solution is applied to the 20 chromatographic columns in the following order: 1) anion exchange; 2) immuno affinity; 3) anion exchange; 4) anion exchange column where Zn^{++} is present in at least the first two steps.

Brief description of the drawings

Fig. 1 illustrates the activation of FVII in the presence of anion exchange columns when varying the Zn^{++} concentration.

Fig. 2 illustrates activation of rFVII to rFVIIa during purification schemes including 5 Zn^{++} in buffers in one or more of the purification steps.

The conditions for scheme A through E are given in table 1 and results are listed in table 2.

Fig. 3 illustrates FVIIa degradation during purification schemes including Zn^{++} in buffers in one or more of the purification steps.

10 The conditions for scheme A through E are given in table 1 and results are listed in table 2.

Prior to setting forth the invention, it may be helpful to an understanding thereof to set forth definitions of certain terms to be used hereinafter:

FVII: FVII will include FVII isolated from plasma or prepared by recombinant DNA-
15 technology. It will also cover allelic variants that may exist and occur from one individual to another and FVII proteins being modified by amino acid residue deletions and/or substitutions which upon activation has the same or substantially the same biological activity for blood coagulation as endogenous human FVIIa.

"FVII" within the above definition will also cover FVII proteins with a variation in
20 degree and location of glycosylation and/or other post-translational modifications depending on the chosen host cells and the cultivation conditions when expressed by recombinant DNA technology.

FVIIa biological activity: The FVIIa biological activity is characterized by the mediation of blood coagulation through the extrinsic pathway.

FVIIa activates FX to FXa, which in turn converts prothrombin to thrombin thereby initiating the formation of a fibrin clot.

5 Controlled activation and degradation: This expression will cover a process in which the activation and degradation of FVII during purification progress according to an intentional controlled variation of a given parameter (in this case the presence of Zn^{++}).

In absence of Zn^{++} , the purification process parameters have to be adjusted to the
10 concomitant occurring activation and degradation reaction. In the presence of Zn^{++} it has turned out to be possible to control these reactions making it possible to optimize purification and activation independently.

Detailed Description

Human FVII will preferably be expressed in transfected mammalian Baby Hamster
15 Kidney (BHK) cells as described in European patent application No. 86302855.1. The culture medium containing FVII and cell growth stimulating proteins will be separated from the cells by centrifugation and filtration prior to the chromatographic purification.

For the purpose of purification FVII will be adsorbed onto different types of
20 chromatographic matrices such as: cat-ion exchangers, an-ion exchangers, immunoaffinity matrices, metal ion chelaters, dye-affinity matrices, hydrophobic interaction matrices and affinity matrices with immobilized bio-specific ligands. The mechanism by which FVII adsorbs to the different matrices varies, hence the effect

of Zn^{++} on the activation of FVII might be different but still significant during adsorption to the different types of chromatographic matrices.

The zinc salt may be added to one or more of the eluate solutions and buffers used for equilibration and elution. The zinc salt may be any soluble zinc salt such as zinc acetate, zinc citrate, zinc chloride or zinc sulphate.

The Zn^{++} concentration may vary between 10 μM and 1 mM and will preferably be between about 20 μM to about 1 mM, more preferably between about 40 μM to about 1 mM.

The subsequent activation of the purified FVII into FVIIa may be achieved using FXIIa as described by Hedner and Kiesel (J.Clin.Invest. 71 (1983), 1836-1841) or with other proteases having trypsin-like specificity (Kiesel and Fujikawa, Behring Inst.Mitt. 73 (1983), 29-42). Alternatively, FVII may be activated in the presence of polymeric macromolecules such as polylysine, matrix structures, substituted agarose gel or membranes.

15 Experimental Part

The purification scheme for purification and activation of rFVII to rFVIIa was performed through the following four successive chromatographic steps: step 1 anion exchange; step 2 immunoaffinity chromatography; step 3 anion exchange; step 4 anion exchange.

20. An experimental series of purification schemes were conducted in which some of the schemes included addition of zinc salt to all the buffers of one or more of the chromatographic steps as outlined in Table 1.

Experimental conditions are given in example 2 to 4.

The reference experiment (E) was performed as example 2 except that no $\text{Zn}(\text{CH}_3\text{-COO})_2$ was added.

Table 1

5

Purification scheme	Chromatographic step			
	1	2	3	4
A	-	+	+	+
B	-	+	+	-
C	-	+	-	-
E (reference)	-	-	-	-

10

+/- indicates the presence or absence of zinc salt in the buffers.

Each step eluate was assayed for the degree of FVII activation (% FVII) and for the content of FVIIa degradation products. The conversion of FVII into the two chained FVIIa form was measured by standard polyacrylamide electrophoresis (PAGE) in sodiumdodecylsulfate (SDS) under reducing conditions combined with Coomassie
15 Blue staining and quantification by laser densitometry.

The content of FVIIa degradation products was quantified by reverse phase high performance liquid chromatography (RP-HPLC) on a butyl bonded silica column in a linear acetonitrile gradient.

The results from experiments conducted essentially as described in the examples
20 are listed in Table 2 and presented in fig. 2 and fig. 3. It is apparent from fig. 2 that in presence of zinc ions (lines A, B and C) none or very limited rFVII activation took

place during the immunoaffinity step (step 2) and the anion exchange steps (step 3 and step 4) (lines A and B).

In contrast hereto an extensive activation took place in step 2 (line E) and in step 3 and 4 (lines B, C and E) in absence of zinc ions.

5 It is also apparent that the interaction of zinc ions with FVII/FVIIa causing the inhibitory effect on the activation of FVII, is of an reversible nature; since after removal of the zinc ions, by complex binding with excess EDTA, FVII is activated during subsequent purification steps (lines B and C).

In step 1 apparently no activation took place in absence of zinc ions. This could be
10 due to a much lower concentration of FVII on the column.

The step 1 eluat, which was used in all the experiments, contained less than 1% FVIIa.

Table 2.

Experiment	Purification steps	Zn ⁺⁺	% FVII (SDS-PAGE)	% FVIIa degraded (RP-HPLC)
A+B	2	+	94	0
	3	+	92	0
5 A	4	+	89	<2
B	4	-	18	3.6
C	2	+	99	<2
	3	-	69	<2
	4	-	2	5.0
E	2	-	46	3.9
	3	-	19	4.6
	4	-	4	8.0

Example 110 Inhibition of the activation of FVII while bound to anion exchanger

The effect of Zn⁺⁺ on the activation of FVII was tested in an experiment in which purified FVII was adsorbed on Q-Sepharose FF matrix in presence of varying concentrations of Zn⁺⁺. 500 µg FVII was incubated with 50 µl Q-Sepharose FF in 800 µl buffer: 10 mM Tris, 50 mM NaCl, 2 mM CaCl₂ and varying concentrations of zinc acetate in 1.5 ml test tubes. After 1 hr and 2 hr the matrix was settled by

centrifugation and the supernatant removed. 800 μ l buffer 10 mM Tris, 50 mM NaCl, 25 mM CaCl_2 pH 8.0 was added and after mixing and centrifugation samples of the supernatant was withdrawn and analyzed for FVII/FVIIa by SDS-PAGE.

The results (fig. 1) showed that in absence of Zn^{++} 55% of the FVII was activated to FVIIa within 1 hr and more than 90% was activated after 2 hr.

In presence of 10 μ M Zn^{++} only 25% was activated after 1 hr. and only 73% was activated after 2 hr.

In presence of Zn^{++} in concentrations above 40 μ M there was no activation within 2 hr.

10 It is clear from this experiment that Zn^{++} in the concentration range of about 10 μ M to about 100 μ M has an inhibitory effect on the activation of FVII in presence of anion exchangers and it is conceivable that Zn^{++} has an effect on FVII activation at all concentrations of Zn^{++} in the whole range from below 10 μ M to 1 mM.

It appears that by varying the Zn^{++} concentration it is possible to control the FVII
15 activation rate constant and at high Zn^{++} concentrations essentially "protect" FVII from activation and hence from degradation.

Example 2

The purification and activation of rFVII to rFVIIa were performed through the following four chromatographic steps:

Step 1:

rFVII containing cell culture medium was adjusted to ion strength below 10 mS/cm by dilution and applied to a Q-Sepharose column pre-equilibrated with buffer A: 10 mM trihydroxymethylaminomethan (Tris); 150 mM NaCl pH 8.

- 5 After a washing step with 175 mM NaCl in the same buffer rFVII was eluted by buffer B: 10 mM Tris; 150 mM NaCl; 25mM CaCl_2 pH 8.

Step 2:

The eluate solution containing 104 mg/l rFVII was adjusted to a final composition: 10 mM Tris; 1M NaCl; 25mM CaCl_2 ; 70 μM $\text{Zn}(\text{CH}_3\text{COO})_2$ pH 7.5 and applied to a
10 Sepharose column with immobilized anti-FVII monoclonal antibody.

The antibody column was pre-equilibrated with buffer C: 10 mM Tris; 100 mM NaCl; 20mM CaCl_2 ; 70 μM $\text{Zn}(\text{CH}_3\text{COO})_2$ pH 7.5. The column was then washed with 10 mM Tris; 2 M NaCl; 20mM CaCl_2 ; 70 μM $\text{Zn}(\text{CH}_3\text{COO})_2$ pH 7.5 followed by buffer C. Thereafter rFVII/rFVIIa was eluted by applying a buffer: 75 mM Tris; 30 mM
15 trisodiumcitrate; 70 μM $\text{Zn}(\text{CH}_3\text{COO})_2$ pH 7.5.

Step 3:

The eluate was immediately applied to a Q-Sepharose column pre-equilibrated with buffer: 10 mM Tris; 150 mM NaCl; 70 μM $\text{Zn}(\text{CH}_3\text{COO})_2$ pH 8.6.

The column was washed with the same buffer and rFVII/rFVIIa was eluted in a linear
20 gradient from buffer A to buffer D: 10 mM Tris; 500 mM NaCl; 70 μM $\text{Zn}(\text{CH}_3\text{COO})_2$ pH 8.6.

Step 4:

The fraction containing rFVII/rFVIIa was adjusted to an ion strength below 10 mS/cm by dilution and immediately applied to a Q-Sepharose column pre-equilibrated with
25 buffer: 10 mM glycylglycine; 150 mM NaCl; 70 μM $\text{Zn}(\text{CH}_3\text{COO})_2$ pH 8.6.

After washing with buffer: 10 mM glycylglycine; 175 mM NaCl; 70 μ M Zn(CH₃COO)₂, pH 8.6; and buffer E: 10 mM glycylglycine; 100 mM NaCl; 70 μ M Zn(CH₃COO)₂, pH 8.6, rFVII/rFVIIa was eluted in a linear gradient from buffer E to buffer: 10 mM - glycylglycine; 100 mM NaCl; 15 mM CaCl₂; 70 μ M Zn(CH₃COO)₂, pH 8.6. The flow rate was 1 vol./hr.

The purified rFVIIa preparation had the following characteristics:

Content of rFVIIa: 345 mg/l measured by UV spectroscopy (OD280)

Content of foreign proteins < 1 % by RP-HPLC

Content of rFVII : 89% by SDS PAGE

10 Content of rFVIIa degradation products: < 2 % by RP- HPLC

Example 3

The purification and activation of rFVII to rFVIIa was performed through the following four chromatographic steps:

Step 1:

15 rFVII containing cell culture medium was adjusted to ion strength below 10 mS/cm by dilution and applied to a Q-Sepharose column pre-equilibrated with buffer A: 10 mM trihydroxymethylaminomethan (Tris); 150 mM NaCl pH 8.

After a washing step with 175 mM NaCl in the same buffer rFVII was eluted by buffer B: 10 mM Tris; 150 mM NaCl; 25mM CaCl₂ pH 8.

Step 2:

The eluate solution containing 104 mg/l rFVII was adjusted to a final composition: 10 mM Tris; 1M NaCl; 25mM CaCl_2 ; 70 μM $\text{Zn}(\text{CH}_3\text{COO})_2$ pH 7.5 and applied to a Sepharose column with immobilized anti-FVII monoclonal antibody.

5 The antibody column was pre-equilibrated with buffer C: 10 mM Tris; 100 mM NaCl; 20mM CaCl_2 ; 70 μM $\text{Zn}(\text{CH}_3\text{COO})_2$ pH 7.5. The column was then washed with 10 mM Tris; 2 M NaCl; 20mM CaCl_2 ; 70 μM $\text{Zn}(\text{CH}_3\text{COO})_2$ pH 7.5 followed by buffer C. Thereafter rFVII/rFVIIa was eluted by applying a buffer: 75 mM Tris; 30 mM trisodiumcitrate; 70 μM $\text{Zn}(\text{CH}_3\text{COO})_2$ pH 7.5.

10 Step 3:

The eluate was immediately applied to a Q-Sepharose column pre-equilibrated with buffer: 10 mM Tris; 150 mM NaCl; 70 μM $\text{Zn}(\text{CH}_3\text{COO})_2$ pH 8.6.

The column was washed with the same buffer and rFVII/rFVIIa was eluted in a linear gradient from buffer A to buffer D: 10 mM Tris; 500 mM NaCl; 70 μM $\text{Zn}(\text{CH}_3\text{COO})_2$

15 pH 8.6.

Step 4:

The fraction containing rFVII/rFVIIa was adjusted to 2 mM ethylenediaminetetraacetic acid (EDTA) and ion strength below 10 mS/cm by dilution and immediately applied to a Q-Sepharose column pre-equilibrated with buffer: 10 mM glycylglycine; 150 mM 20 NaCl pH 8.6.

After washing with buffer: 10 mM glycylglycine; 175 mM NaCl pH 8.6. and buffer E: 10 mM glycylglycine; 100 mM NaCl pH 8.6, rFVII/rFVIIa was eluted in a linear gradient from buffer E to buffer: 10 mM glycylglycine; 100 mM NaCl; 15 mM CaCl_2 pH 8.6. The flow rate was 1 vol./hr.

The purified rFVIIa preparation had the following characteristics:

Content of rFVIIa: 492 mg/l measured by UV spectroscopy (OD280)

Content of foreign proteins < 1 % by RP-HPLC

Content of rFVII : 18 % by SDS PAGE

5 Content of r FVIIa degradation products: 3.6 % by RP-HPLC

Example 4

The purification and activation of rFVII to rFVIIa was performed through the following four chromatographic steps.

Step 1:

10 rFVII containing cell culture medium was adjusted to ion strength below 10 mS/cm by dilution and applied to a Q-Sepharose column pre-equilibrated with buffer A: 10 mM trihydroxymethylaminomethan (Tris); 150 mM NaCl pH 8.

After a washing step with 175 mM NaCl in the same buffer rFVII is eluted by buffer B: 10 mM Tris; 150 mM NaCl; 25mM CaCl₂ pH 8.

15 Step 2:

The eluate solution containing 104 mg/l rFVII was adjusted to a final composition: 10 mM Tris; 1M NaCl; 25mM CaCl₂; 70 μM Zn(CH₃COO)₂ pH 7.5 and applied to a Sepharose column with immobilized anti-FVII monoclonal antibody.

The antibody column was pre equilibrated with buffer C: 10 mM Tris; 100 mM NaCl; 20 20mM CaCl₂; 70 μM Zn(CH₃COO)₂ pH 7.5. The column was then washed with 10 mM Tris; 2 M NaCl; 20mM CaCl₂; 70 μM Zn(CH₃COO)₂ pH 7.5 followed by buffer C.

Thereafter rFVII/rFVIIa was eluted by applying a buffer: 75 mM Tris; 30 mM trisodiumcitrate; 70 μ M $\text{Zn}(\text{CH}_3\text{COO})_2$ pH 7.5.

Step 3:

The eluate was adjusted to 2 mM EDTA and immediately applied to a Q-Sepharose 5 column pre-equilibrated with buffer: 10 mM Tris; 150 mM NaCl pH 8.6.

The column was washed with the same buffer and rFVII/rFVIIa was eluted in a linear gradient from buffer A to buffer D: 10 mM Tris; 500 mM NaCl pH 8.6.

Step 4:

The fraction containing rFVII/rFVIIa was adjusted to ion strength below 10 mS/cm by 10 dilution and immediately applied to a Q-Sepharose column pre-equilibrated with buffer: 10 mM glycylglycine; 150 mM NaCl pH 8.6.

After washing with buffer: 10 mM glycylglycine; 175 mM NaCl pH 8.6. and buffer E: 10 mM glycylglycine; 100 mM NaCl pH 8.6, rFVII/rFVIIa was eluted in a linear gradient from buffer E to buffer: 10 mM glycylglycine; 100 mM NaCl; 15 mM CaCl_2 pH 8.6. The flow rate was 1 vol./hr.

The purified rFVIIa preparation had the following characteristics:

Content of rFVIIa: 1.2 mg/ml measured by UV spectroscopy (OD280)

Content of foreign proteins < 1 % by RP-HPLC

20 Content of rFVII : 1 % by SDS-PAGE

Content of rFVIIa degradation products: 6.7 % by RP-HPLC

CLAIMS

1. A process for controlled activation and degradation of Factor VII during purification whereby a solution of Factor VII is subjected to a number of chromatographic purification steps wherein Zn^{++} is present at least in one of the 5 purification steps.
2. A process according to claim 1, wherein a solution of Factor VII is applied to a number of anion exchange and immuno affinity chromatography columns.
3. A process according to claim 1 - 2, wherein Zn^{++} is present in the form of a soluble zinc salt.
- 10 4. A process according to claim 1 - 3, wherein Zn is present in a concentration of from about 10 μM to about 1 mM.
5. A process according to claim 4, wherein Zn^{++} is present in a concentration of from about 20 μM to about 1 mM.
6. A process according to claim 4, wherein Zn^{++} is present in a concentration of 15 from about 40 μM to about 1 mM.
7. A process according to claim 2, wherein the FVII solution is applied to the chromatographic columns in the following order: 1) anion exchange; 2) immuno affinity; 3) anion exchange; 4) anion exchange column and wherein Zn^{++} is present in at least two steps.

1/3

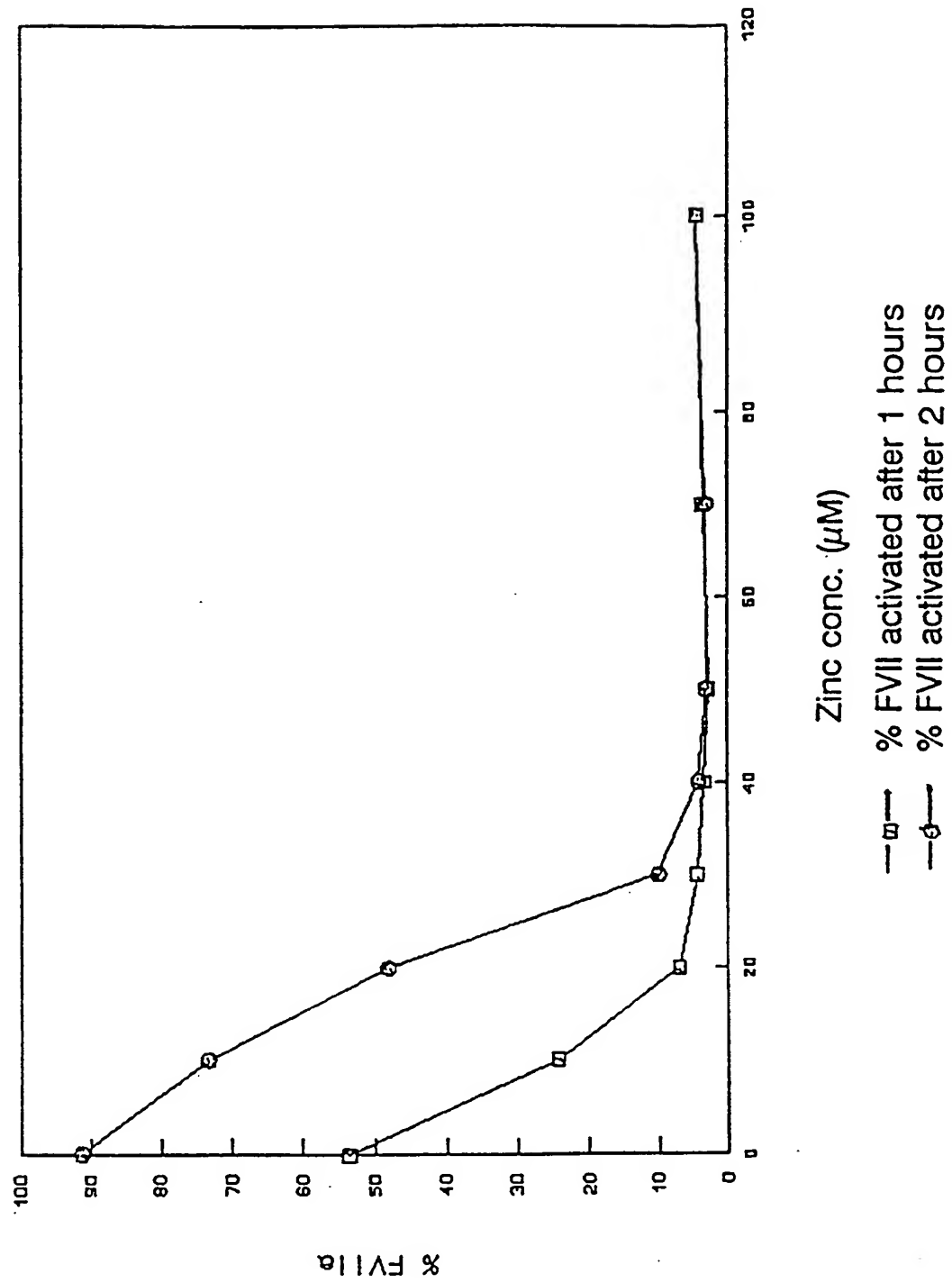


Fig. 1

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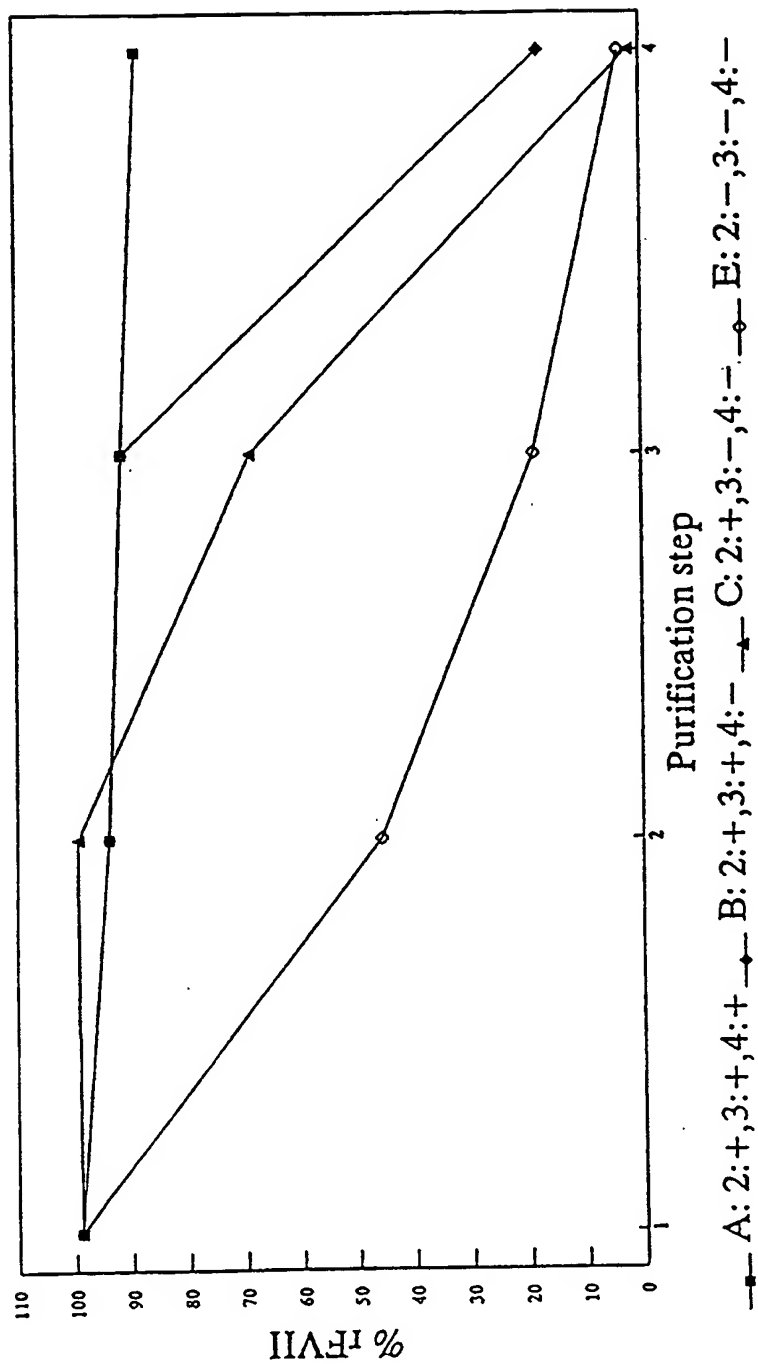


Fig. 2

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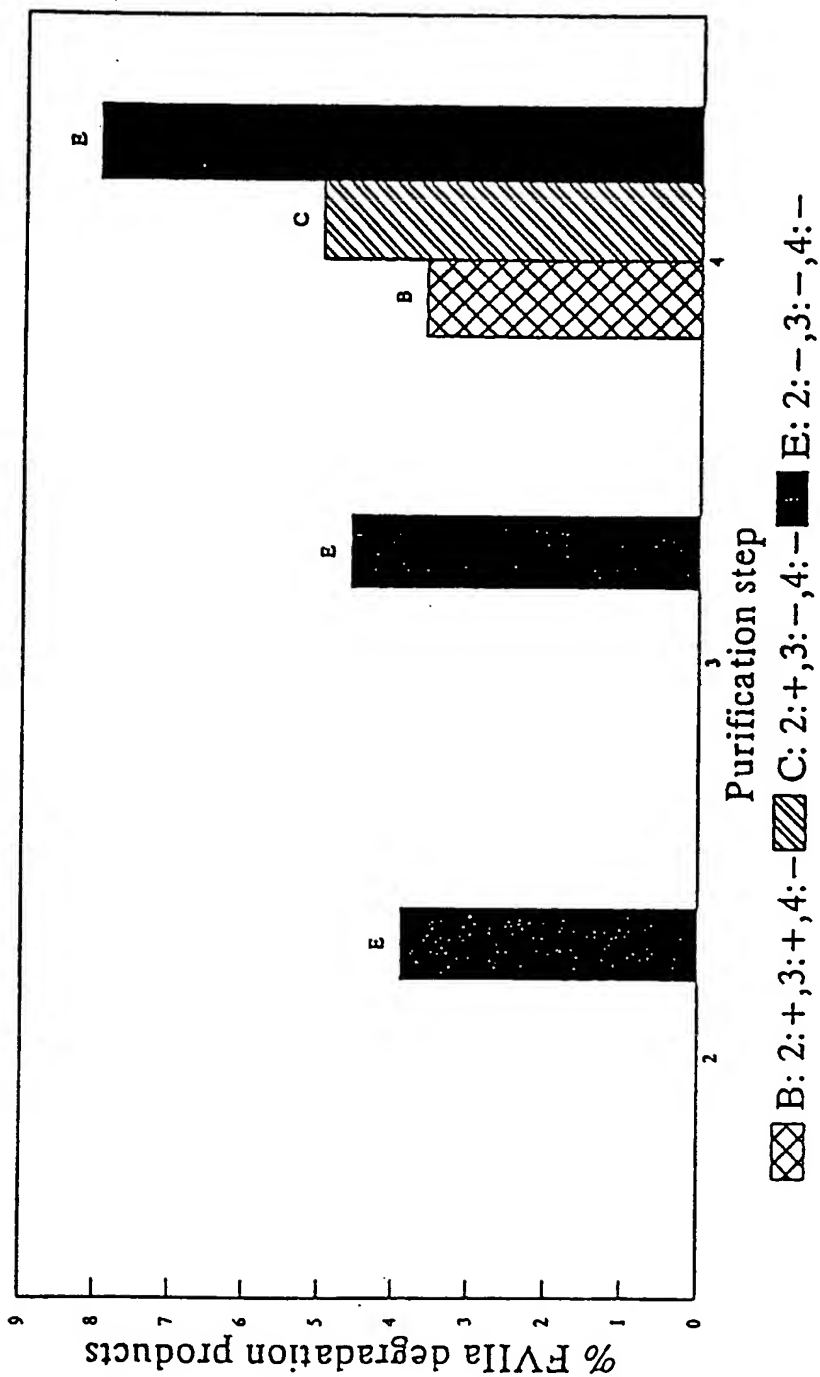


Fig. 3

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 94/00122

A. CLASSIFICATION OF SUBJECT MATTER

IPC5: C07K 3/22, C07K 3/28, A61K 35/16

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC5: A61K, C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

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C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Dialog Information Services, File 155, Medline, Dialog accession no. 07816391, Medline accession no. 91335391, Pedersen AH. et al: "Inhibition of recombinant human blood coagulation factor VIIa amidolytic and proteolytic activity by zino ions". Thromb Haemost (GERMANY) May 6 1991, 65 (5) p 528-34 --	1-7
A	Dialog Information Services, File 155, Medline, Dialog accession no. 02688481, Medline accession no. 75095481, Radcliffe R: "Activation and control of factor VII by activated factor X and thrombin. Isolation and characterization of a single chain form of factor VII. J Biol Chem (UNITED STATES) Jan 25 1975, 250 (2) p 388-95 --	1-7

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

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Facsimile No. +46 8 666 02 86

Authorized officer

Carolina Palmcrantz

Telephone No. +46 8 782 25 00

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP, A2, 0363126 (ELI LILLY AND COMPANY), 11 April 1990 (11.04.90), page 2, line 46 - page 3, line 3; page 3, line 53, the claims -- -----	1-7

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